Supplementary Information

Combinatorial targeting and discovery of ligand-receptors in organelles of mammalian cells

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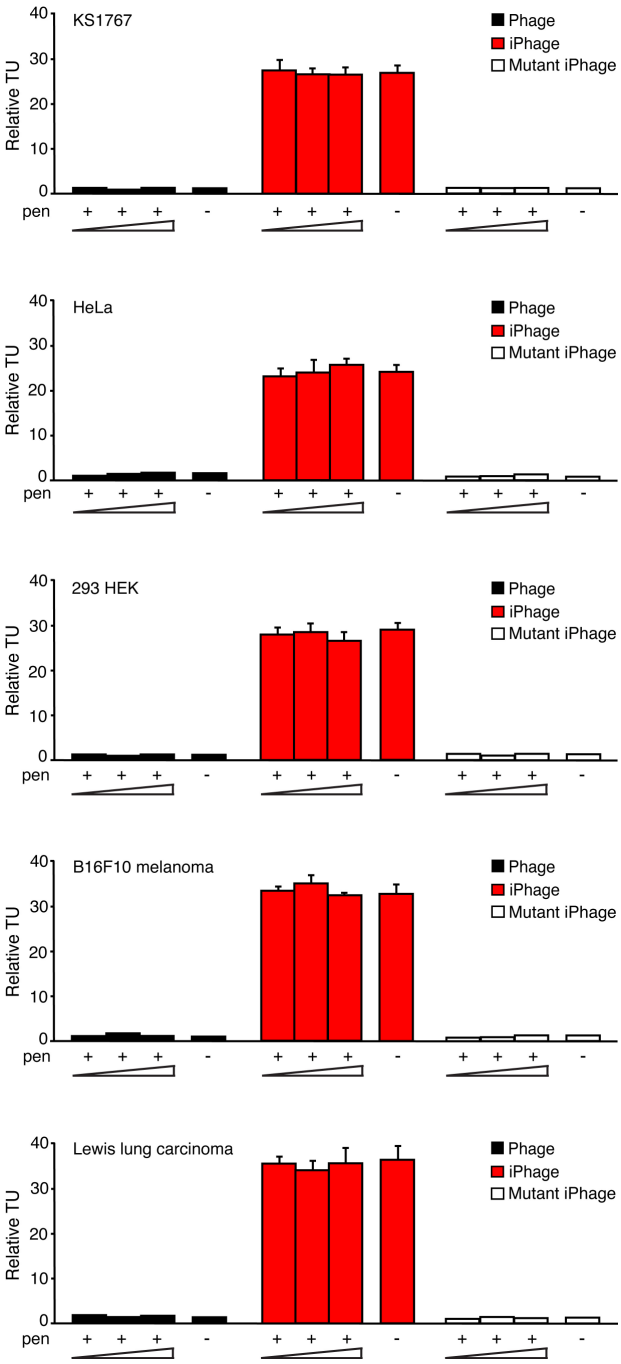
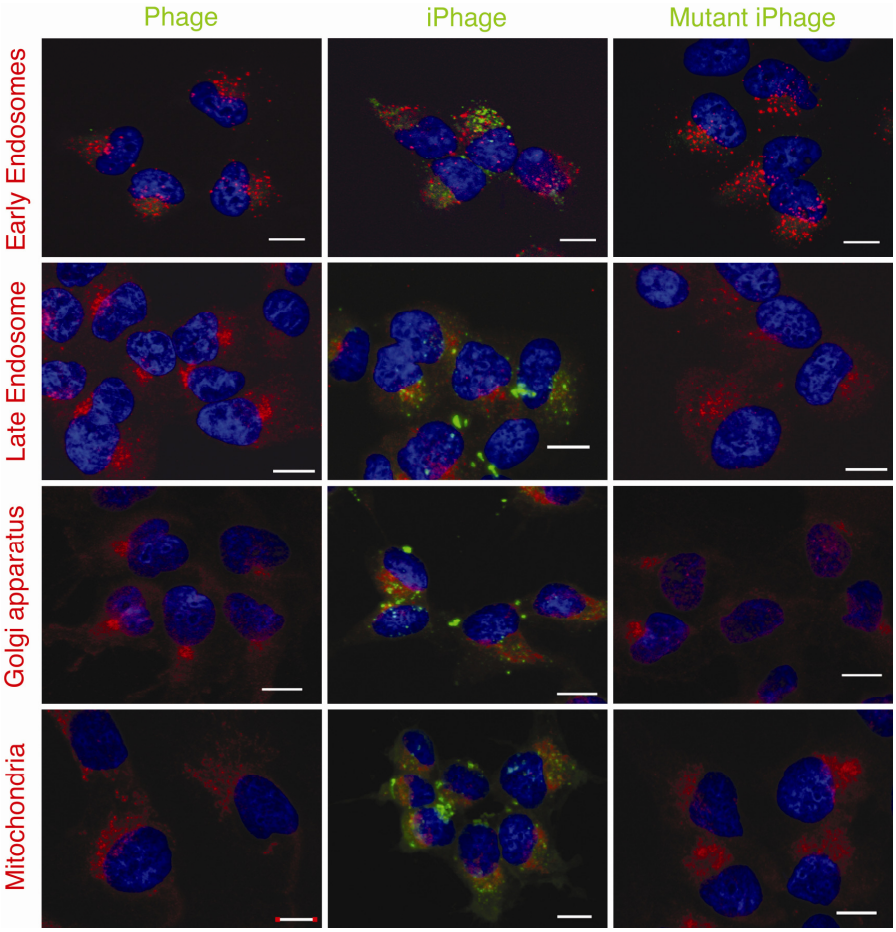
Supplementary Information contains:

Supplementary Figures S1-S8

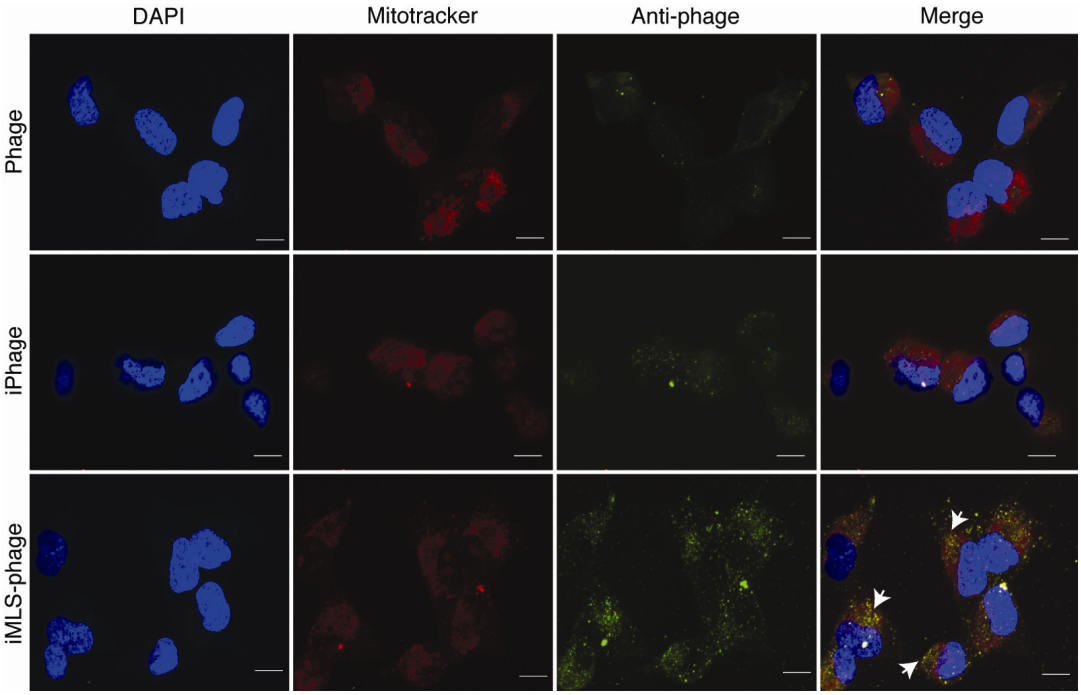
Supplementary Methods



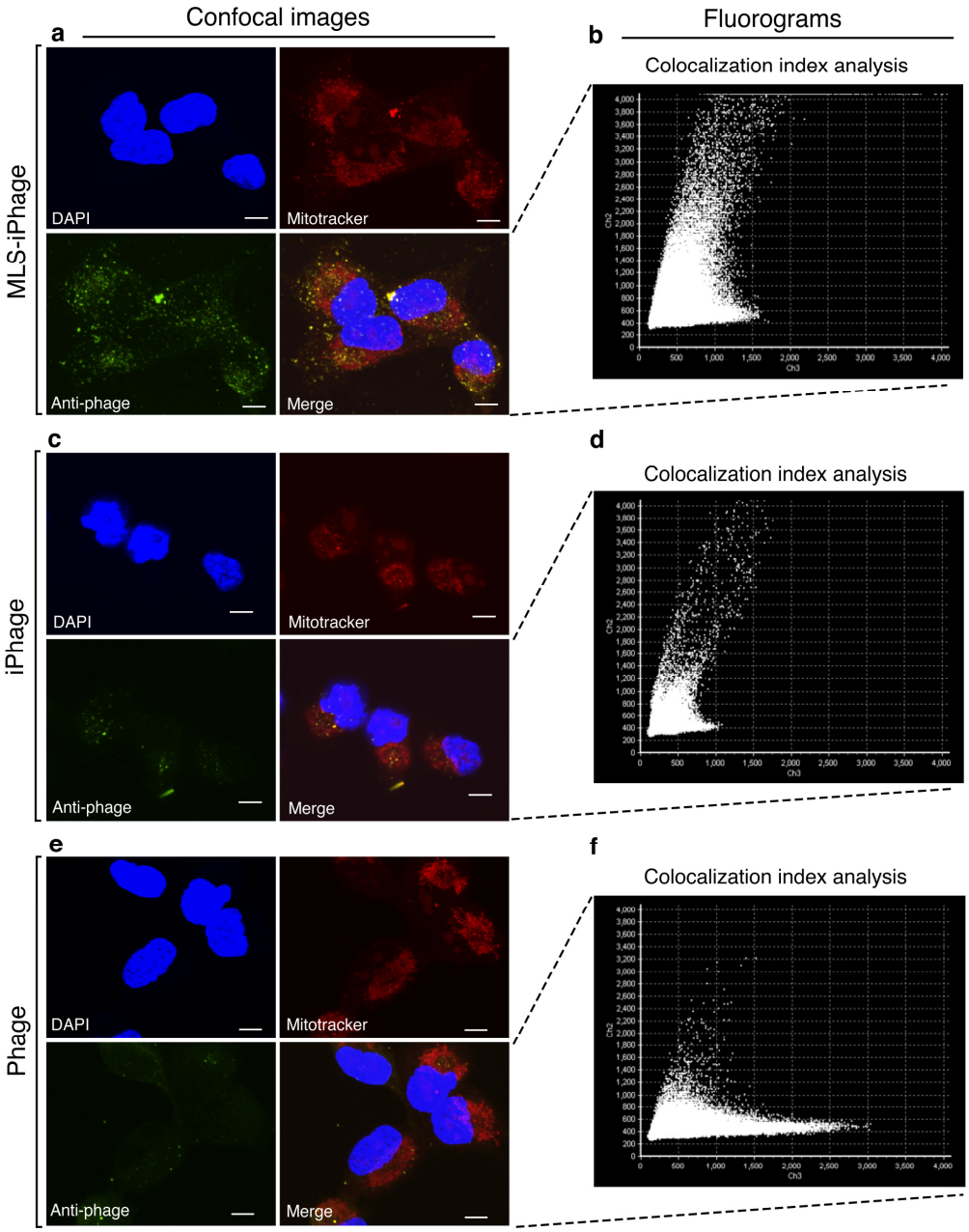
Supplementary Figure S1| iPhage internalization was not inhibited with penetratin (pen) peptide in different mouse and human cell types. Cells were preincubated with pen peptide (10, 30, 100 μM), and incubated with phage particles for 16 h at 37°C. Bars represent mean values for phage transducing units (TU) recovered from the cytosol fraction ± SEM, from triplicates.



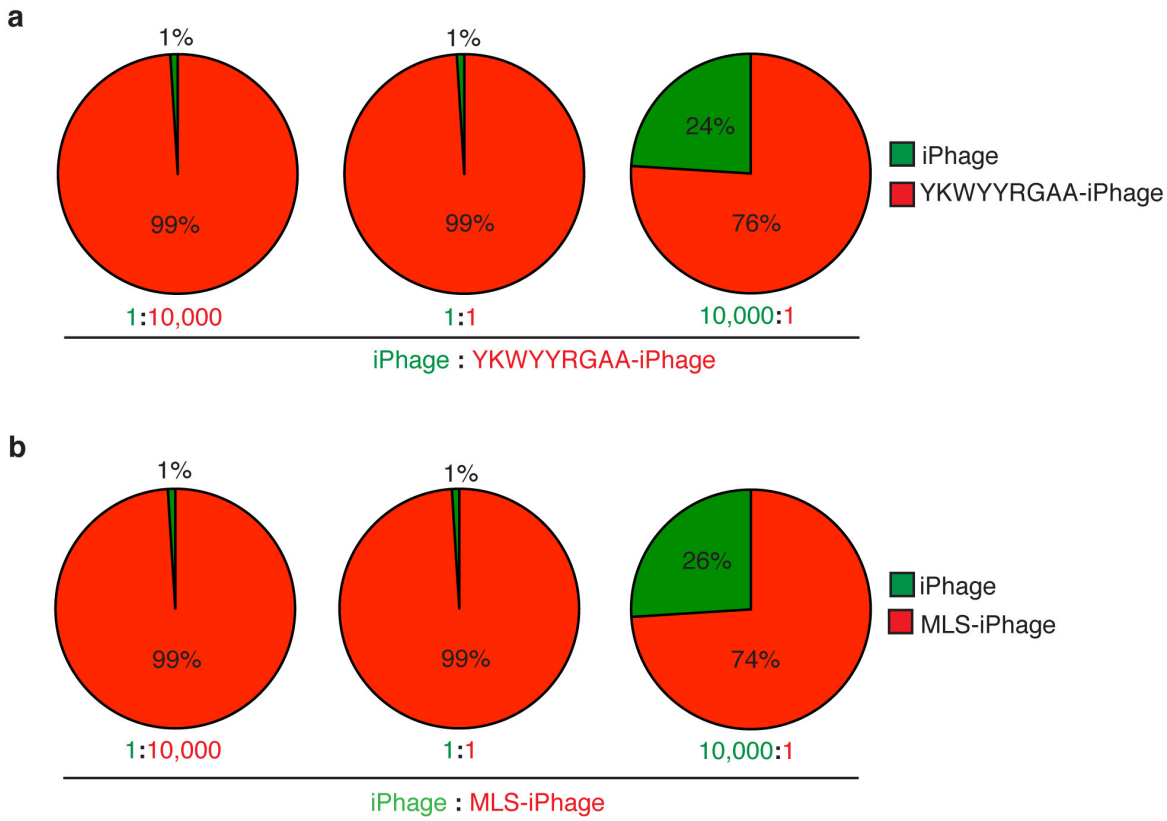
Supplementary Figure S2| iPhage particles were distributed evenly in the cytosol. The KS1767 cells were incubated with phage, iPhage and mutant iPhage for 24 h at 37ºC. Intracellular distribution of phage particles within the cytosol were detected by using anti-phage (green fluorescence), and anti-organelle (red fluorescence) antibodies. Subcellular markers used: anti-early endosome antigen-1 (EEA1), anti-mannose 6 phophate receptor [M6PR, (late endosome)], anti-58K (Golgi apparatus), and anti-ERAB [hydroxysteroid (17-beta) dehydrogenase 10; mitochondria]. DAPI counterstain nucleus (blue fluorescence). Scale bar, 10 µm.



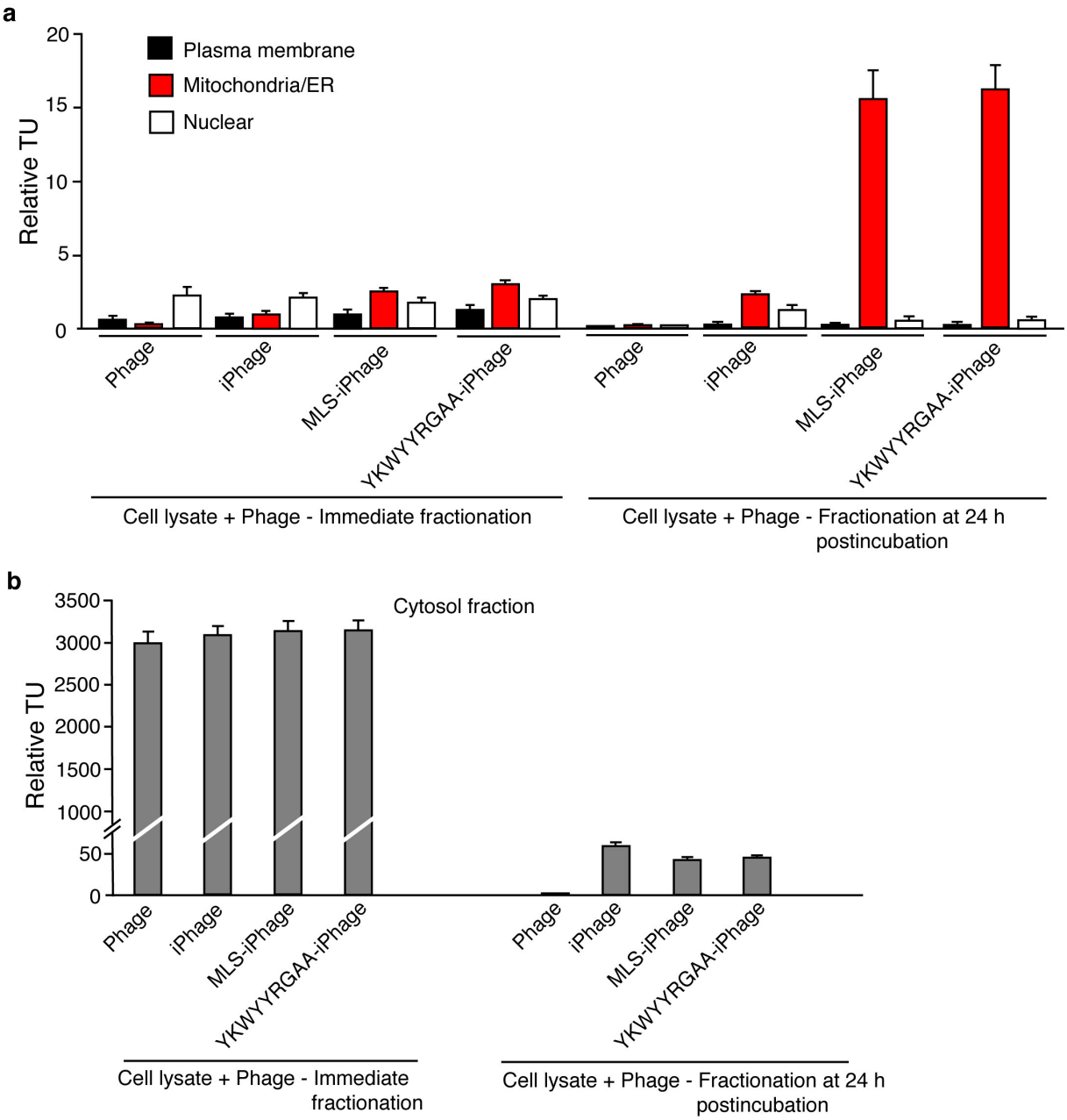
Supplementary Figure S3| Confocal fluorescence microscopy analysis of KS1767 cells exposed to parental phage, iPhage and iMLS-phage. Intracellular localization was revealed by anti-rabbit Alexa Fluor 488, Orange-Mitotracker (200 nM/ 25 min), and DAPI counterstain. Scale bar, 10 µm. Arrows indicate signal co-localization.



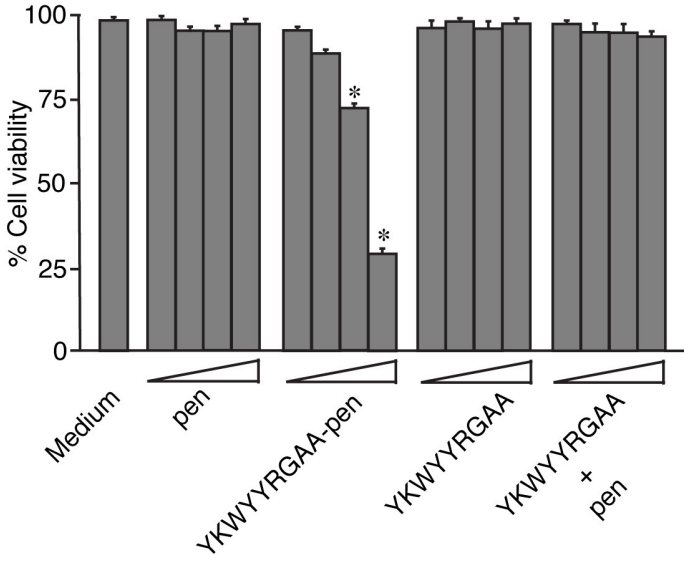
Supplementary Figure S4| Colocalization index analysis of MLS-iPhage versus controls. Images were analyzed as a complete z-stack series with the Olympus FV10-ASW V31 co-localization software. The fluorograms compared the colocalization of the phage-FITC (Channel 2) on the x-axis with the mitotracker-RFP (Channel 3) on the Y-axis. Confocal images of MLS-iPhage (**a**), iPhage (**c**), phage (**e**) and fluorograms respectively (**b**, **d**, **f**). The co-localization index for this data indicates 10- and 1000-fold more co-localization in the MLS-iPhage compared to iPhage and phage respectively. Scale bar, 10 µm.



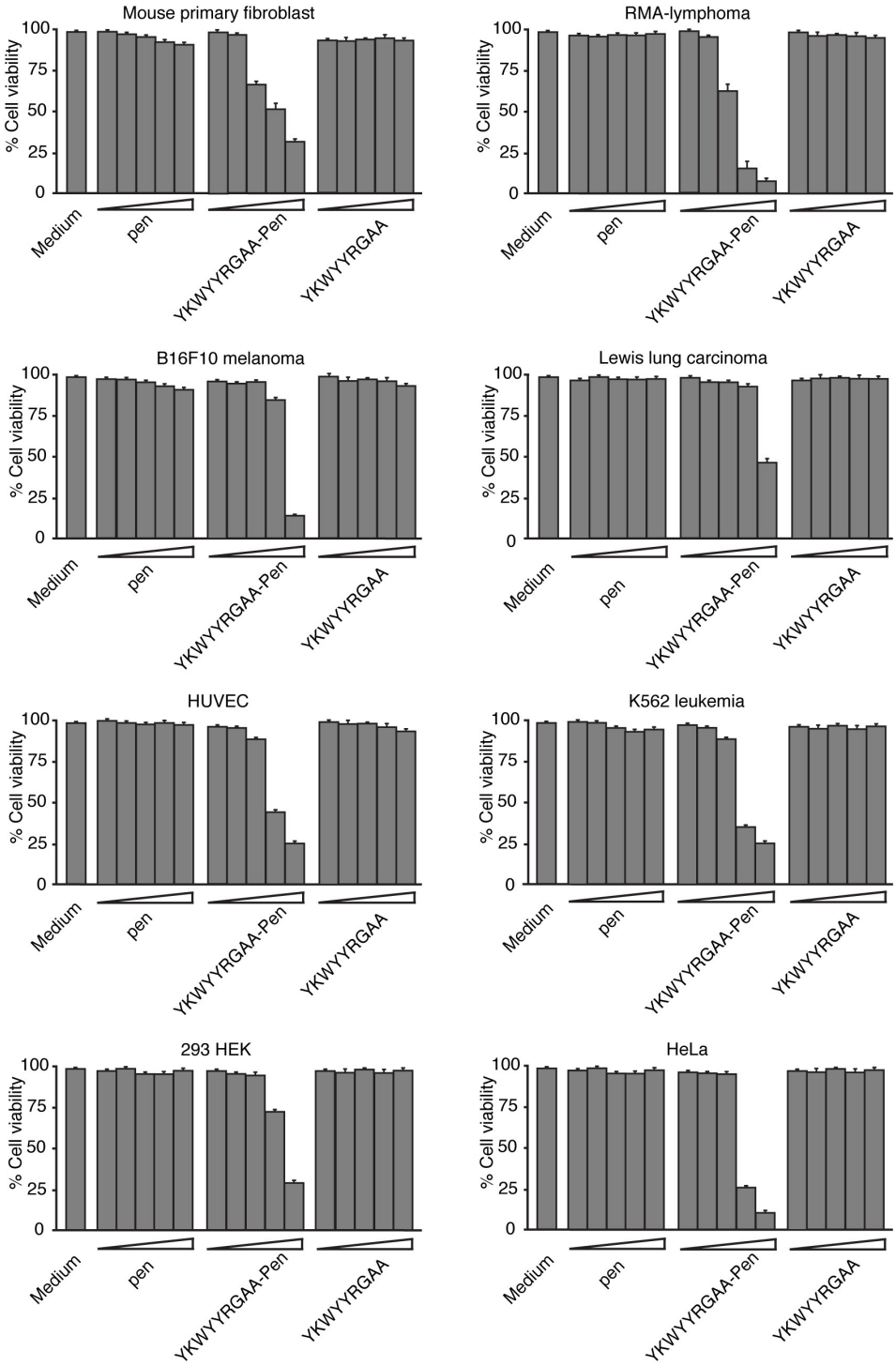
Supplementary Figure S5| Targeting frequency of YKWYYRGAA/or MLS-iPhage clone versus iPhage particles in the mitochondria fraction. The YKWYYRGAA-iPhage (**a**) and MLS-iPhage (**b**) clones were enriched in the mitochondria/ER fraction when they were mixed to equal or 10,000-fold more iPhage particles. KS1767 cells were superimposed with different admixtures of phage particles overnight at 37ºC [i.e., 10^5 TU of iPhage + 10^9 TU of MLS-iPhage (1:10,000), 10^7 TU of iPhage + 10^7 TU of MLS-iPhage (1:1), 10^9 TU of iPhage + 10^5 TU of MLS-iPhage (10,000:1)]. Mitochondria/ER fractions were isolated for phage recovery by K91 bacteria infection. The pie charts represent the percentage (%) frequency of sequences randomly selected from single bacteria colonies.



Supplementary Figure S6| The MLS and YKWYYRGAA iPhage clones were enriched in the mitochondria/ER fractions after 24 h post-incubation. (**a**) Plasma membrane, nucleus and mitochondria/ER fractions were isolated either immediately or 24 h post-incubation with phage particles. (**b**) Cytosol fraction was used as a control of phage viability and internalization for immediate and 24 h post-incubation respectively. Bars represent mean values for transducing units (TU) recovered from the subcellular fractions ± SEM, from triplicates.



Supplementary Figure S7| Cell viability was reduced upon exposure of YKWYYRGAA-pen, relative to controls (1, 3, 10 and 30 μM). Undetected synergist cell death effects were detected in non-conjugated admixtures of pen plus YKWYYRGAA peptides. The symbol * indicates significant reduction of cell viability ($P < 0.0001$). The MTT assay was performed in triplicate. Bars represent mean ± SEM. Data were statistically analyzed by Student's *t*-test.



Supplementary Figure S8| Induction of cell death in different murine and human cell types upon exposure to YKWYYRGAA-pen peptide. Cells were plated on a 96-well plate and were exposed to each peptide (1, 3, 10, 30 and 100 μM) for 6 h at 37°C. The MTT assay was performed in triplicate. Bars represent mean ± SEM.

SUPPLEMENTARY METHODS

Reagents. The following antibodies were obtained from commercial sources: anti-EAA1, anti-M6RP, anti-ERAB, and anti-Golgi antibodies (Abcam).

Membrane and cytosol fractionation. KS1767 cells were incubated with 5 x 10^9 TU of the Phage, iPhage and mutant-iPhage overnight at 37 °C. The next day, cells were washed extensively with pre-warmed PBS and were subsequently detached with trypsin. Cells were washed with ice-cold PBS, incubated with hypotonic buffer [10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5)] for 15 min, and placed in a standard Dounce homogenizer to disrupt cell membranes. Then, we added stabilization buffer [525 mM mannitol, 175 nM sucrose, 2.5 mM EDTA (pH 7.5), and 12.5 mM Tris-HCl (pH 7.5)]. The organelle suspension was centrifuged at 1,300 g for 5 min at 4 °C, the supernatant was transferred to a new tube, and centrifuged at 17,000 g for 15 min; this pellet contained the mitochondria/ER enriched fraction. Next, the supernatant was centrifugated 100,000 g for 30 min at 4°C. The pellet contained the enriched plasma membrane and the supernatant the cytosol fraction. The subcellular fraction-bound phage population was recovered through infection of logarithmic growth phase of k91kan *Escherichia coli (E. coli)* for 1 h at room temperature. Serial dilutions of the infected bacteria were plated on Luria-Bertani (LB) plates containing tetracycline (40 mg/ml) and kanamycin (100 mg/ml) to determine the recovery phage titer.

Intracellular iPhage competition assay. KS1767 cells cultured in 6-well plate and incubated with increasing concentrations of iPhage (10^5, 10^7, 10^9 TU) were mixed with decreasing

concentrations of YKWYYRGAA or MLS-iPhage (10^9, 10^7, 10^5 TU) respectively [i.e., 10^5 iPhage + MLS-iPhage 10^9 (1:10,000), 10^7 iPhage + 10^7 MLS-iPhage (1:1), 10^9 iPhage + MLS-iPhage 10^5 (10,000:1)]. After 24 h incubation, cells were washed, trypsinized and rinsed with ice-cold PBS. Then, cells were incubated with hypotonic buffer [10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5)] for 15 min, and placed in a standard dounce homogenizer to disrupt cell membranes. Next, we added stabilization buffer [525 mM mannitol, 175 nM sucrose, 2.5 mM EDTA (pH 7.5), and 12.5 mM Tris-HCl (pH 7.5)]. The organelle suspension was centrifuged at 1,300 g for 5 min at 4 °C, the supernate was transferred to a new tube, and centrifuged at 17,000 g for 15 min; this pellet contained the mitochondria/ER enriched fraction. The subcellular fraction-bound phage population was recovered through infection of log-phase k91kan *E. coli* for 1 h at room temperature. Serial dilutions of the infected bacteria were plated on LB plates containing tetracycline (40 mg/ml) and kanamycin (100 mg/ml) to obtain single bacteria clones. Finally, we randomly picked 96 colonies per competition assay, each clone was phage-PCR and DNA sequenced.

DNA fragmentation assay. KS1767 cells were cultured in 96-well plate and exposed to different peptides (30 μM) or media as a negative control. The microplate was incubated for 4 h at 37ºC and 5% CO_2. Next, we used the cell death detection ELISA plus and followed the manufacturers recommendations (Roche). The assay was run in triplicate.

Protein Expression and Purification. The recombinant GST-fusion expressing ribosomal protein L29 (RPL29) was generated by PCR cloning, with forward and reverse primers respectively (5'-CACAGAATTCATGGCGAAGTCCAAGAACCACACC-3';

5'-CACAGCGGCCGCCTACTCTGAAGCCTTTGTAGGGGCCTGG-3'). Five nanograms of plasmid (human RPL29 clone, Invitrogen) were used as a template in a 50 µl PCR reaction with high fidelity Taq polymerase (Stratagene). The cycling parameters consisted of one cycle of 94 ºC for 3 min, 35 cycles of 94 ºC for 0.5 min, 60 ºC for 1 min, and 68 ºC for 1 min, followed by a single 10 min cycle at 72 ºC for extension. The PCR product was purified on Qiagen columns, and both plasmid (pGEX4T-1; Amersham) and PCR product were digested with *Eco*RI and *Not* I restriction enzymes, gel-purified, and ligated overnight at 16 °C. Ligated products were precipitated in ethanol and electroporated into DH5α *E. coli*; these bacteria were plated on LB agar with carbenicillin (50 mg/ml). GST-RPL29 and GST expressing plasmids were transformed into BL21 *E. coli* for recombinant protein expression. In brief, BL21 *E. coli* were cultured in rich liquid media (2XYT) supplemented with 50 mg/ml of carbenicillin until an OD_{590nm} ~0.6 was reached, at this point GST expression was induced with 1 mM IPTG. After overnight incubation at 30 °C, bacteria were collected by centrifugation (5,000 g for 10 min) and lysed with Bugbuster reagent (EMD Biosciences, CA). Purified recombinant proteins were analyzed by staining an SDS-PAGE gel with Coomassie blue, and by Western blotting using an anti-GST antibody (Amersham).

Western blot. Total cell extracts from KS1767 cells treated with or without peptides, were resuspended in radio immuno precipitation assay (RIPA) lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100], supplemented with protease inhibitor mixture (Roche). The protein concentration of the cell lysates was determined by the Bradford method (BioRad). Lysates were electrophoretically resolved on 4%-20% gradient sodium dodecyl sulfate polyacrylamide gels (BioRad), and transferred to nitrocellulose

membranes. The membranes were blocked and incubated with primary antibodies diluted 1:200 in blocking buffer [5% milk in 0.1% Tween-20, 100 mM NaCl, 10mM Tris-HCl, (pH 7.4)]. Membranes were washed and incubated with secondary antibodies anti-rabbit IgG HRP (1:2000). Reactive bands were visualized using the enhanced chemiluminescence reagent system (ECL; Amersham).

Southern blot. KS1767 cells were grown in 6-well tissue-plate and incubated with 10^9 transducing units (TU) of iPhage, mutant iPhage, and parental phage in MEM supplemented with 1% FBS at 37 °C. After 24 h incubation, the cells were washed, and trypsinized. The genomic DNA was extracted by using a commercial kit (QIAGEN DNeasy blood & tissue kit). The DNA was analyzed in a 0.8% agarose gel and treated with acidic (0.25 M HCl), denaturing (0.5N NaOH; 1M NaCl) and neutralizing [0.5M Tris (pH 7.4); 1.5M NaCl] solutions. Next, genomic DNA was transferred into a nylon membrane by overnight capillarity using 10X saline-sodium citrate (SSC) buffer. The membrane was UV crosslinked and washed with 2X SSC buffer for 5 min. Then, we generated a phage genomic probe by using the PCR primers forward 5'-TTTATA CGGGCACTGTTACTCAAG-3' and reverse 5'-TTTCATCGGCATTTTCGGTCATAG-3'. After PCR amplification, the 322 bp fragment was gel purified (QIAquick, QIAGEN) and radiolabeled using a commercial kit (Megaprime DNA labeling kit, Amersham). Finally, the nylon membrane containing the genomic DNA was hybridized with ^{32}P-probe, washed under stringent conditions and exposed to X-ray film overnight at -80ºC for 24 h.

Transmission Electron Microscopy. Cells were fixed with a solution containing 3% glutaraldehyde plus 2% PFA in 0.1 M cacodylate buffer (pH 7.3) for 1 h. After fixation, the

samples were washed, treated with 0.1% Millipore-filter cacodylate-buffered tannic acid, and post-fixed with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated and embedded in LX-112 medium, and polymerized in a 70°C oven for 2 days. Ultra-thin sections were cut on an ultracut microtome (Leica), stained with uranyl acetate and lead citrate in a Leica EM stainer and examined on a JEM 1010 transmission electron microscope (JEOL). Digital images were captured at an accelerating voltage of 80 kV (Advanced Microscopy Techniques Corp).